

Effect of Different Extraction Methods on Vitamin B₁₂ from Blue Green Algae, *Spirulina Platensis*

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Abstract

In the present study different methods of extraction of vitamin B₁₂ from *Spirulina are* compared. Six different extraction procedures were carried out and their effects of on total vitamin B₁₂ and the form of vitamin B₁₂ are presented. The results of the extraction method using KCN for the total vitamin B₁₂ and aqueous extraction for identifying the true form of vitamin B₁₂ in *Spirulina* are reproducible. The use of cyanide converted all forms of vitamin B₁₂ to a stable cyanocobalamin. Aqueous extraction helped in identifying the true form of vitamin B₁₂ in the algae. Presences of vitamin B₁₂ in the sample were compared by HPLC, microbiological, chemiluminescence and MS/MS methods. The results showed that the method of extraction influences the content of vitamin B₁₂. Cyanocobalamin, a stable form of vitamin B₁₂ was quantified in *Spirulina* using microbiological assay, chemiluminescence assay and gold nanoparticle based RNA aptamer analysis and found correlation among these methods.

Keywords: *Spirulina Platensis*; Vitamin B₁₂ extraction; Microbiological method; Chemiluminescence; Aunps Based RNA aptamer

Introduction

Vitamin B₁₂ is an important cofactor in many biochemical reactions and was identified nearly 80 years ago as the anti-pernicious anemia factor in liver [1]. Vitamin B₁₂ contains a family of derivatives with some forms being active and others not active in humans. The active form of cobalamin is of significant interest in human nutrition since this cannot be obtained from pure plant foods. This particular vitamin is essential for normal maturation and development of blood cells (erythrocytes). The ultimate source of all Vitamin B₁₂ is through microbial production. This vitamin is produced industrially by microbiological means using different strains of *Propionibacterium* and *Pseudomonas* [2]. Plants do not contain cobalamin since they have no cobalamin dependent enzymes [3]. Hence strict vegetarians are at greater risk of vitamin B₁₂ deficiency. On the other hand many algae are reported to be rich in vitamin B₁₂ [4]. Some species such as *Porphyra yezoensis* contains as much cobalamin as liver and was also shown to supply adequate amount of bioavailable vitamin B₁₂ when consumed by strict vegetarians [4-6]. Although plants and animals cannot synthesize or store Vitamin B₁₂, it is effectively accumulated and recycled in these organisms through the food chain.

Food and cell extracts may contain variety of cobalamins including cyanocobalamin, hydroxocobalamin, methylcobalamin and adenosylcobalamin [7]. The predominant cobalamin forms in meat are adenosylcobalamin and hydroxocobalamin where as dairy products primarily contains methylcobalamin and hydroxocobalamin. But the algal vitamin B₁₂ is not completely elucidated. Vitamin B₁₂ in algae is either in true form or analog form and the activity of these forms is not been studied.

Because of the importance of this vitamin, many extraction methods have been developed but a standardized protocol for its extraction from algae is not yet studied. Many methods have been devised to extract vitamin B₁₂ from microorganisms [8]. However the matrix in algae is different from other organisms and the food samples. Hence a standardized protocol is required for extraction of B₁₂ from the algal samples.

This paper discusses various extraction methods for vitamin B₁₂. The different extraction methods are compared and vitamin B₁₂ present in *Spirulina* has been quantified.

Materials and Methods

Chemicals and Instruments:

Cyanocobalamin (CN-Cbl), hydroxocobalamin (OH-Cbl), adenosylcobalamin (Adl-Cbl) and methylcobalamin (Me-Cbl), luminol and urea hydrogen peroxide, diethylpyrocarbonate (DEPC), silver nitrate, gold (III) chloride and trisodium citrate were from Sigma Aldrich (Bangalore, India). Amberlite XAD-2 was procured from supelco sigma Aldrich (Bangalore, India). Vitamin B₁₂ assay medium was obtained from Himedia, Bangalore, India. RNA aptamer sequence (5' GGA ACC GGU GCG CAU AAC CAC CUC AGU GCG AGC AA 3') was adapted from Lorsch and Szostak report [9]. Pyrimidines were 2' fluoro modified and obtained from Trilink Biotechnologies (San Diego CA, USA). DEPC treated water was used for preparing stock solution. All reagents used were of analytical grade and methanol was of HPLC grade. Ammonium pyrrolidine dithiocarbamate from Himedia. UV-visible Spectrophotometer (UV-160A) and HPLC (SCL-10-AVP) were procured from Shimadzu Kyoto, Japan. HPLC C-18 Column (4.6*300mm, μ bondpack, particle size 10μm) was procured from Waters (USA). Luminometer is from Luminoskan TL plus, thermolab systems, Finland. Data acquisition was performed with decimal HyperTerminal TL plus software. The column used for electrospray ionization mass spectrometry was Acquity UPLC HSS T3, 50 ×2.1 mm, 1.8 μm. positive ion tandem mass spectrometry

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experiments were performed in product mode on a triple quadrupole TQD mass spectrometer (Waters Corporation, Milford, MA).

Organism and culture

Spirulina platensis CFTRI strain was used for the experimental purpose. The algal cells were aseptically cultured in modified Zarrouk's medium [10] at 25 °C ± 2°C using shaker at 40 rpm. The *S. platensis* culture was grown at ambient temperature in raceway pond. The culture growth was measured in terms of optical density at 560nm. The biomass was harvested by gravity filtration and washed twice with distilled water. The harvested biomass was lyophilized and used for analysis.

Extraction of Vitamin B₁₂

Method 1: Freeze dried biomass of *Spirulina* was treated with acetate buffer pH 4.8. Total B₁₂ was extracted from the cell suspension by boiling with 20mg KCN for 30 min at 98 °C. The extract was centrifuged at 1000 g for 10min and the supernatant was analyzed for vitamin B₁₂. [11].

Method 2: Freeze dried biomass was taken in sodium acetate buffer pH 4.0. To this sodium cyanide (1.0%) and 0.25g of α -amylase were added while stirring and the solution was incubated at 42°C for 30 min. After adjusting the pH to 4.8, the solution was heated at 98°C for 35min. The extract was cooled and centrifuged at 1000 g for 10 min and the supernatant was analysed for vitamin B₁₂ [12].

Method 3: Freeze dried biomass sample was extracted using 0.5% Ammonium Pyrrolidine Dithiocarbamate (APDC) and 2% citric acid in 50% DMSO and incubated on shaker for 45 min at 55°C. The sample was cooled and centrifuged. Supernatant was analyzed for vitamin B₁₂ [13].

Method 4: Freeze dried biomass was treated with 80% ethanol. The suspension was boiled for 20 min at 80°C. The extract was centrifuged at 1000 g for 10min. The supernatant was evaporated under vacuum and used for vitamin B₁₂ analysis.

Method 5: Freeze dried biomass of *Spirulina platensis* was suspended in triple distilled water. Vitamin B₁₂ was extracted by autoclaving at 121°C for 10 min. The homogenate was centrifuged at 1000 g for 10min. The cooled supernatant was adjusted to pH 6.0 and used for the B₁₂ analysis [14].

Method 6: This method includes extraction using benzyl alcohol and chloroform as reported by Rudking and Taylor [15]. Freeze dried biomass was first treated with 1% potassium cyanide and pH was adjusted to 9.5 to 10.0. The sample was allowed to stand for 5 hours at room temperature. Sodium sulfate (20% w/v) was added to the solution. The pH was further adjusted to 11.0 with sodium hydroxide and aqueous solution was extracted three times using one tenth volume of benzyl alcohol. To the combined benzyl alcohol extracts one half volume of chloroform was added and the solvent phase was extracted three times with one tenth volumes of water. The extract so obtained was analyzed for vitamin B₁₂. All the extraction procedures were carried out in dark.

Purification

The vitamin B₁₂ extracts obtained were concentrated before the purification process using Amberlite XAD-2. Amberlite XAD-2 column was prepared as a methanolic suspension of the resin packed to a bed height of 15-16 cm. The column was equilibrated with water. The sample was loaded onto the column and was eluted with 80 % (v/v) methanol. The eluant was concentrated using Rotavapor (Buchi).

The concentrate was further purified by passing through C18 Sep-Pak column and eluted with 25% ethanol.

High performance liquid chromatography

The purified sample was injected on to HPLC which was equipped with C-18 column (4.6*300 mm, μ bondpack, particle size 10 μ m) with a PDA detector. The absorbance was measured at 361 nm and 546 nm. The solvent used were (A) 50% methanol with 0.1% acetic acid (B) water, with a flow rate of 1ml /min for 40min. The vitamin B₁₂ compound was eluted with a linear gradient of methanol [from 0% to 90% of a 50% (v/v) methanol solution] [16]. The retention times (RT) of authentic standards of OH-Cbl, CN-Cbl, Adl-Cbl and Me-Cbl were recorded.

Assay of Vitamin B₁₂ using *E. coli*

The *E.coli* (ATCC 11105) strain was grown in maintenance medium at 37°C and mixed with B₁₂ assay agar and pour plated. Cups, 5 mm in diameter, were bored in the solid agar medium. Standard vitamin B₁₂ and purified sample (50 μ l) were inoculated into the cups. Triple distilled water was used as control and the plates were incubated at 37°C for 24 hrs. Zone of growth was observed after 24 hrs.

Assay of Vitamin B₁₂ using *Lactobacillus delbrueckii*

Vitamin B₁₂ was assayed by the microbiological method using *Lactobacillus delbrueckii* MTCC 911 strain obtained from Microbial Type Culture Collection, CSIR-IMTECH, Chandigarh, India. The standard vitamin B₁₂ was prepared using distilled water within the range of 0.01-0.2 μ g/ml for the analysis. Purified *Spirulina* sample was analysed. The turbidity (%T) of *Lactobacillus delbrueckii* test culture was measured at 600nm using Shimadzu spectrophotometer (UV-160A) [17].

Chemiluminescence assay

Luminometer was used for chemiluminescence (CL) reactions. The CL reactions were done in a polystyrene cuvette, and the CL signals were plotted at 10-s intervals for a period of 10 min. Urea-H₂O₂ was added to a mixture of optimized concentration of luminol and vitamin B₁₂ in the cuvette. This resulted in CL signals, generated due to reaction between luminol and urea hydrogen peroxide with vitamin B₁₂ [18]. The generation of signals was measured in terms of chemiluminescence units (CLU) An increase in CLU can be observed in the presence of vitamin B₁₂.

Analysis of vitamin B₁₂ by MS/MS

The electron spray ionization mass spectrum (ESI-MS) was performed in positive mode using linear gradient of 10mM ammonium formate and 0.1% formic acid (A) and 10 mM ammonium formate and 0.1% formic acid in methanol (B) as mobile phase. The cone gas at 28 L/h and desolvation gas at 1000 L/h was set. Samples were introduced into the mass spectrometer through a direct flow injection system for solvent delivery at the flow rate of 0.6 ml/min. Capillary and cone voltage were 3.00 kV and 28 V respectively. Source temperature 120°C, desolvation at 400 °C. The column temperature was set at 35°C. MS/MS of sample and standard was recorded.

Gold nanoparticle and RNA aptamer detection

For the detection of vitamin B₁₂, RNA aptamer-based colorimetric sensor using gold nanoparticles (AUNPs) were employed. A mixture consisting of an optimized concentration AUNPs and aptamer was gently shaken for 10 mins at room temperature. Purified sample from method 1 was added and incubated for 10 mins. NaCl was added slowly

to the incubated sample. The change in colour and spectra were recorded by ultraviolet-visible (UV-vis) spectrophotometer [19].

Results and Discussion

Measurement of vitamin B₁₂ concentration in food has presented enormous analytical challenge as it is complex, highly sensitive and water soluble molecule. Extracting vitamin B₁₂ from algal material with all the other metabolite complicates an already difficult separation process. Another major obstacle to vitamin B₁₂ analysis is the amount of vitamin B₁₂ present in the organism which is generally low in quantity.

Many test materials may contain hydroxocobalamin, adenosylcobalamin, methylcobalamin. However they may be lost during the extraction procedure due to their unstable nature. Inclusion of cyanide before heating converts these forms to the more stable cyanocobalamin form. The addition of cyanide also aids extraction by converting the various coenzyme forms to cyanocobalamin. Addition of cyanide will hinder identification of different forms of vitamin B₁₂ present in the sample. Though it is one of the most reliable methods of extraction, identification of forms of vitamin B₁₂ in the sample needs to be standardized. Therefore in the present study cyanide extraction was compared with other extraction methods to find a suitable method for identifying different vitamin B₁₂ forms present.

Many water immiscible solvents were tested and proposed for extraction, concentration and purification of vitamin B₁₂ from natural materials. This includes extraction with benzyl alcohol and chloroform method (Rudking and Taylor, 1952) which is used for fermentable broth.

Many solvents were used for extraction of vitamin, but in each case certain disadvantages made them undesirable for experimental work. Probably the best solvent is water which can extract many of the water soluble materials. Hence extraction using water as solvent was tried along with ethanol and DMSO.

The algal sample consists of many other components that cause chromatographic interference with vitamins. Therefore the sample extracted was purified using Amberlite XAD-2 and Sep-Pak C18 cartridges. Use of these columns helps in efficient binding of the vitamin B₁₂ from the complex matrix and enabled separation of water soluble vitamins and removed most of the interfering components [20]. The purified sample was collected and evaporated to dryness and analyzed

HPLC analysis of vitamin B₁₂

HPLC analysis for vitamin B₁₂ was carried out using a suitable solvent system for separating different forms of vitamin B₁₂. Retention time of standard OH-Cbl, CN-Cbl, Adl-Cbl and Me-Cbl were found to be 20.1, 25.1, 29.2 and 35.2 mins respectively. The extraction procedure in which cyanide was added i.e method 1 and 2 the retention time of the sample was similar to that of the standard cyanocobalamin (Figure 1A and Figure 2A). However in the chromatogram of the sample from method 2 there were interfering peaks. In the methods 3 and 4, the RT of the sample were not comparable to any of the forms of vitamin B₁₂ studied (Figure 2B and Figure 2C). The RT of the peak from Method 5 resembled to that of standard methylcobalamin (Figure 3A and Figure 3B). No peak was observed in the extract from method 6.

Chemiluminescence analysis of vitamin B₁₂

The purified samples were analyzed for cobalt-enhanced chemiluminescence. It is reported that Co²⁺ enhances the photon production during the luminal and H₂O₂ reaction. During the

chemiluminescence reaction, photons are produced that are directly proportional to vitamin B₁₂ concentration. Chemiluminescence can detect Co²⁺ of both true and pseudo forms. All the samples extracted in different methods showed enhancement of cobalt indicating the

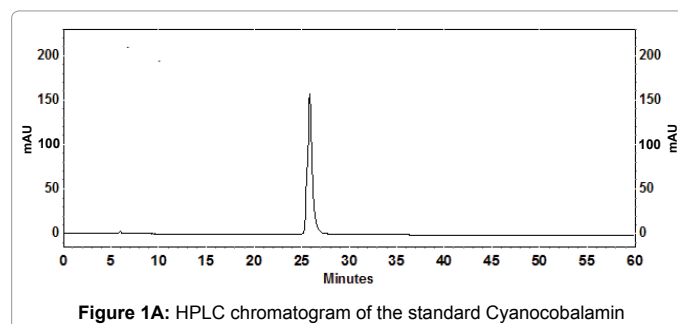


Figure 1A: HPLC chromatogram of the standard Cyanocobalamin

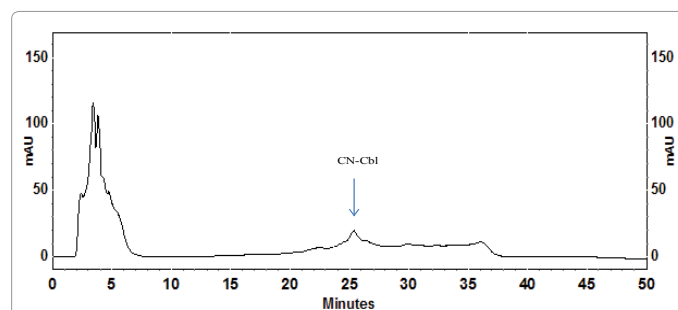


Figure 1B: HPLC chromatogram of the *Spirulina* extract showing peak similar to that of the cyanocobalamin

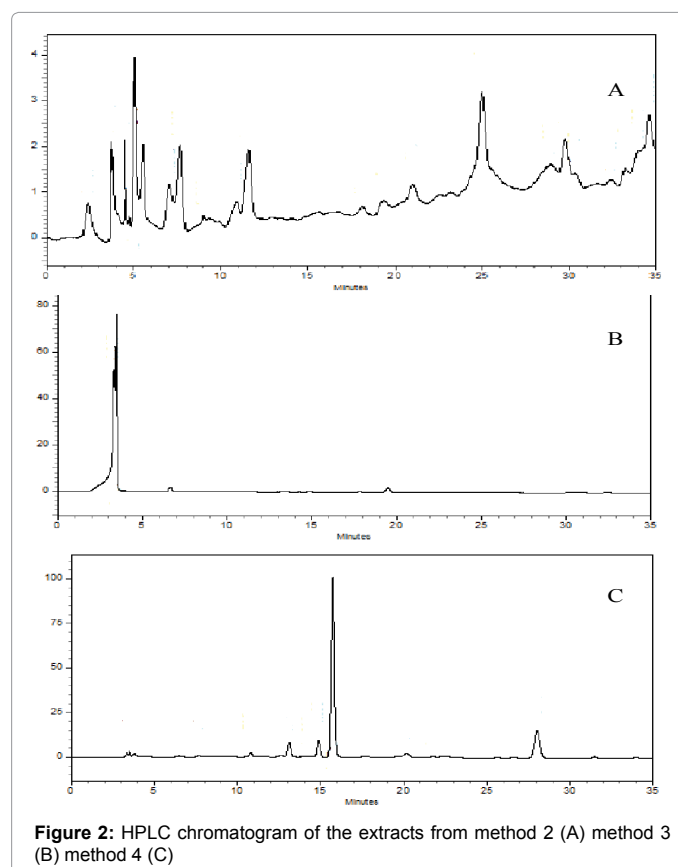


Figure 2: HPLC chromatogram of the extracts from method 2 (A) method 3 (B) method 4 (C)

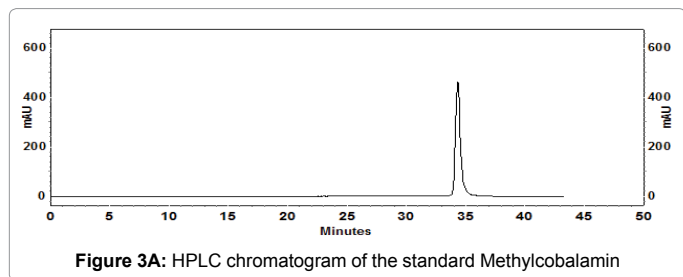


Figure 3A: HPLC chromatogram of the standard Methylcobalamin

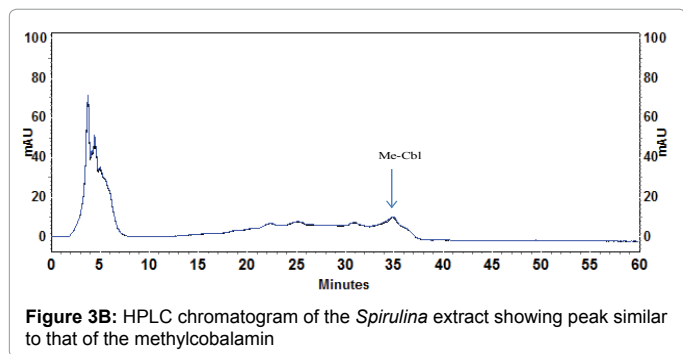


Figure 3B: HPLC chromatogram of the *Spirulina* extract showing peak similar to that of the methylcobalamin

Method of extraction	Total Vitamin B12 content in <i>Spirulina</i> biomass (µg/100g) by microbiological assay	Total Vitamin B12 content in <i>Spirulina</i> biomass (µg/100g) by Chemiluminescence assay
Method 1	220±1.8	213±0.4
Method 2	142±0.9	207±1.7
Method 3	116±0.8	65±0.9
Method 4	460±6.2	211±2.2
Method 5	184±1.4	190±0.5

Table 1: Quantification of vitamin B₁₂ by Microbiological method using *Lactobacillus delbrueckii*

Algae	Microbiological Assay (µg/100g)	Chemiluminescence (µg/100g)	Gold nanoparticle based aptamer method (µg/100g)
Cyanocobalamin	220±1.8	213±0.4	190±0.2

Table 2. Quantification of total cyanocobalamin in *Spirulina* by different methods

presence of cobalamin in all the extracts. HPLC analysis of extract from method 6 did not show any peak corresponding to standard cobalamins, while analysis by chemiluminescence showed presence of cobalamin. This difference between the methods could be due to the sensitivity of the chemiluminescence assay upto picogram level while in HPLC it is microgram.

Microbiological Assay of vitamin B₁₂

To confirm the presence of vitamin B₁₂ in the algae, the purified samples were subjected to microbiological assay using *E.coli*. A zone of *E.coli* growth was observed surrounding the wells containing standard vitamin B₁₂ as well as for the purified algal samples obtained using methods 1, 2, 3 and 5 confirming the presence of vitamin B₁₂. Algal extract obtained by method 4 and 6 did not support any growth of microbes. The use of solvents such as ethanol, benzyl alcohol and chloroform during extraction procedure in the method 4 and 6 might have inhibited the growth of microbes in the assay. There was no microbial growth surrounding the wells of the control containing triple-distilled water.

Quantifying vitamin B₁₂ using microbiological assay using *Lactobacillus delbrueckii* is one of the most frequently applied method for routine analysis of vitamin B₁₂. The microbial growth measured in terms of turbidity of the cell broth is proportional to vitamin B₁₂ concentration. The quantification of vitamin B₁₂ in the purified *Spirulina* sample was done through microbiological assay using *Lactobacillus delbrueckii* MTCC 911 (Table 1). The amount of vitamin B₁₂ was found to vary from 110 to 400 µg for 100 g dry biomass of *Spirulina platensis*.

Quantification of total vitamin B₁₂ using gold nanoparticle (AUNPs) based RNA Aptamer

Aptamers are short DNA or RNA sequences that have high affinity towards its analyte. Aptamer using AUNPs have been considered as one of the highly specific and sensitive on-site detection method [21]. AUNPs solutions are red in color because of their specific and size dependent surface plasmon resonance (SPR) absorption at 520 nm. When salt was added to the solution containing AUNPs, vitamin B₁₂ aptamer and the sample, the AUNPs get aggregated and led to a red-to-purple color change and an additional absorption peak at 640nm. UV-vis studies provided quantitative results that clearly showed absorption at 520 nm gradually decreased while absorption at 640 nm increased. The blue shift in the surface plasma Resonance absorption suggested the formation of large AUNPs aggregates. Based on the spectral data, total cyanocobalamin in the sample obtained from method 1 was found to be 190 ± 0.2/100g dry weight. Total cyanocobalamin was quantified using microbiological, chemiluminescence and goldnanoparticle based RNA aptamer (Table 2).

MS/MS of Vitamin B₁₂ in the extract

The purified extracts from method 1 and 5 showed correlation in quantification among the methods followed and hence these extract were further analyzed using MS/MS. MS/MS analysis of method 1, showed the presence of M+H cyanocobalamin i.e. 1356 (Figure 4A) and the purified extract of method 5 showed the presence of doubly charged fragments of methylcobalamin. The mass of methylcobalamin is *m/z* 1344.38. The spectrum shows that it is doubly charged and a mass of 673.31 was observed (Figure 4B).

Of all the extraction protocols followed, method 1 and method 5 showed a good correlation in quantifying vitamin B₁₂ between the microbiological and chemiluminescence assay methods, whereas the other methods of extraction showed 2-3 fold difference between microbiological and chemiluminescence assays. This difference in quantification may be because of some interfering compounds which might be present in the extracts (Method 2-4 and 6). The results indicated that the method of extraction influences the content of vitamin B₁₂. Method 5 which involved aqueous extraction appears to be an excellent method for identifying forms of vitamin B₁₂, it is less labor-intensive, and is simpler and faster than other methods.

In the present study six different extraction protocol have been compared and all the extraction were some of the most frequently used procedure for the extraction of vitamin B₁₂ in different food materials. The recovery of above ninety percent (92-95%) in method 1 and 2, 83% in method 3, while 88 to 90% in methods 4 and 5 and least (70%) in method 6 were observed. Method 1 which involves the addition of KCN is one of the most general methods employed for the extraction of vitamin B₁₂. Method 2 and 3 was used for the extraction of bound vitamin B₁₂ from food, premixes and pharmaceutical products respectively. Method 4 and 5 were used for the water soluble vitamins

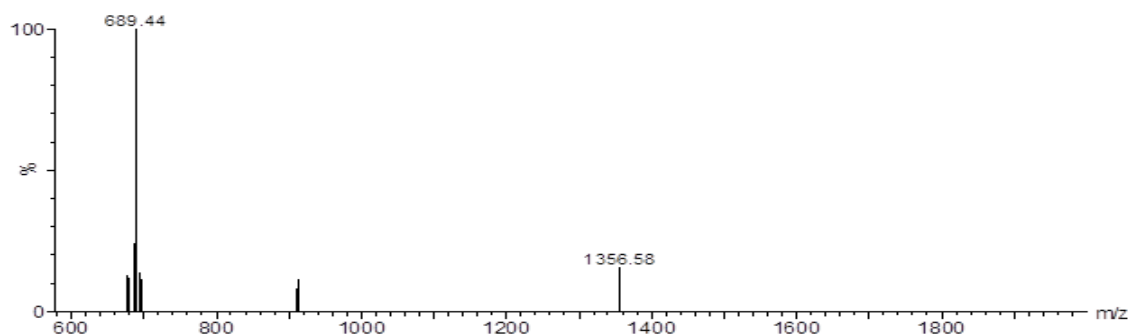


Figure 4A: Mass spectrum Cyanocobalamin in *Spirulina* extract.

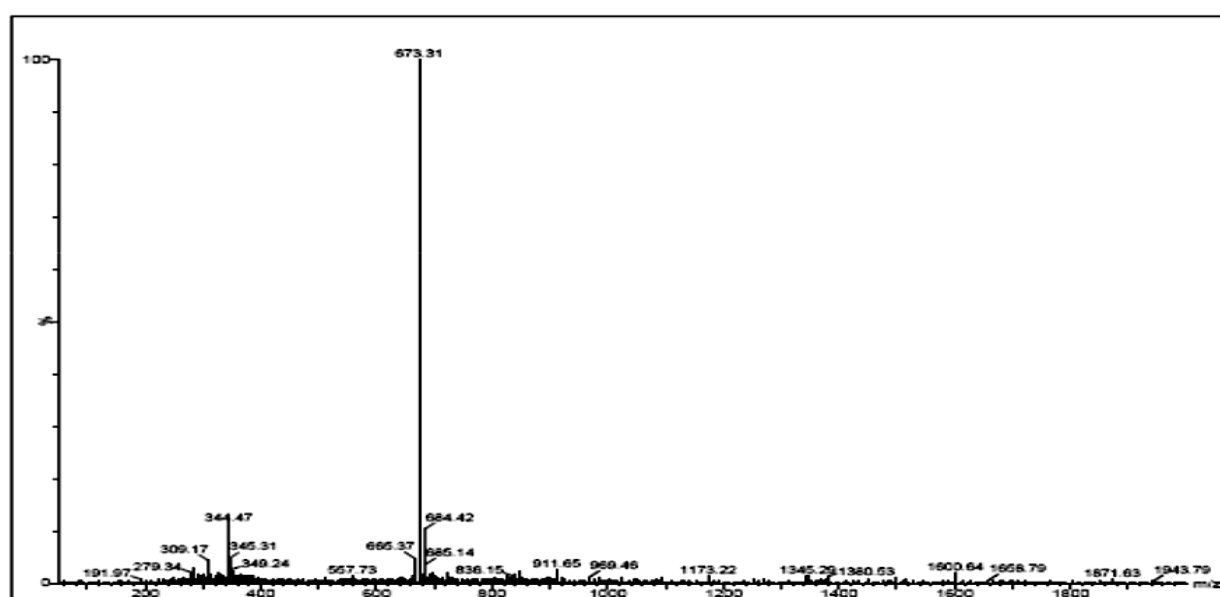


Figure 4B: Mass spectrum of Methylcobalamin in *Spirulina* extract.

while method 6 is one of the chemical methods for determining vitamin B₁₂ in fermentation broths. Each method had its limitation when it comes for the extraction in algae since, there are constraints in analyzing the vitamin B₁₂ from algae. The amount of vitamin B₁₂ present is low. Secondly the alga contains many important metabolites which will form a matrix and extracting a pure form of vitamin B₁₂ will be a challenging task. Addition of cyanide helps in conversion of all forms of B₁₂ to cyanocobalamin which hinders the identification of the forms of vitamin B₁₂ present in algae. The extraction method with ethanol showed high values of vitamin B₁₂ which is possibly due to co-eluting metabolites. DMSO method of extraction does not involve the addition of cyanide and hence the peak corresponding to that of cyanocobalamin was not found. Method 6 of Rudking and Taylor [15] is not suitable for algal samples, as repeated extraction dilutes the amount of the vitamin B₁₂ and hence the quantification was not possible in the present study (data not shown in table 1). The recovery of each method was evaluated using standard vitamin B₁₂.

Conclusion

Different extraction protocol for vitamin B₁₂ has been compared in algae for the first time. The method of extraction greatly influences

the content and the forms of vitamin B₁₂. In conclusion, most suitable extraction methods for the vitamin B₁₂ could be the one using potassium cyanide where the results can be reported as the “total vitamin B₁₂” and aqueous extraction method where the form of vitamin B₁₂ present can be identified. Since algae can be alternative source of vitamin B₁₂ among vegetarians these finding can facilitates the extraction in different algae and also identify the forms of vitamin B₁₂ in algae.

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